



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

## The Impact of the Eda Pathway on Tooth Root Development

**Citation for published version:**

Fons Romero, JM, Star, H, Lav, R, Watkins, S, Harrison, M, Hovorakova, M, Headon, D & Tucker, AS 2017, 'The Impact of the Eda Pathway on Tooth Root Development', *Journal of Dental Research*, vol. 96, no. 11, pp. 1290-1297. <https://doi.org/10.1177/0022034517725692>

**Digital Object Identifier (DOI):**

[10.1177/0022034517725692](https://doi.org/10.1177/0022034517725692)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Peer reviewed version

**Published In:**

Journal of Dental Research

**General rights**

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

**Take down policy**

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



## The impact of the Eda pathway on root development

Journal:	<i>Journal of Dental Research</i>
Manuscript ID	JDR-17-0304.R1
Manuscript Type:	Research Reports
Date Submitted by the Author:	n/a
Complete List of Authors:	Fons, Juan; Kings College London, Craniofacial Development Star, Haza; Kings College London, Craniofacial Development Lav, Rupali; Kings College London, Craniofacial Development Watkins, Sophie; Guy's & St Thomas' NHS Foundation Trust, Hypodontia Clinic Harrison, Mike; Guy's & St Thomas' NHS Foundation Trust, Hypodontia Clinic Hovorakova, Maria; Institute of Experimental Medicine AS CR, Department of Teratology Headon, Denis; The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, United Kingdom , The Roslin Institute Tucker, Abigail; Kings College London, Craniofacial Development
Keywords:	Cell signalling, Craniofacial anomalies, Dental morphology, Developmental Biology, Epithelial-mesenchymal interactions, Gene expression
Abstract:	The Eda pathway (Eda, Edar, Edaradd) plays an important role in tooth development, determining tooth number, crown shape and enamel formation. Here we show that the Eda pathway also plays a key role in root development. Edar (the receptor) is expressed in Hertwig's Epithelial Root sheath (HERS) during root development with mutant mice showing a high incidence of taurodontism: large pulp chambers lacking or showing delayed bifurcation or trifurcation of the roots. The mouse upper second molars in the Eda pathway mutants show the highest incidence of taurodontism, this enhanced susceptibility being matched in human patients with mutations in EDA-A1. These taurodont teeth form due to defects in the direction of extension of the HERS from the crown, associated with a more extensive area of proliferation of the neighbouring root mesenchyme. In those teeth where the angle at which the HERS extends from the crown is very wide and therefore more vertical, the mutant HERS fail to reach each other in the normal furcation region and a taurodont tooth is created. The phenotype is variable, however, with milder changes in angle and proliferation leading to normal or delayed furcation. This is the first analysis of the role of Eda in the root, showing a direct role for this pathway during postnatal mouse development, and suggests that changes in proliferation and angle of HERS may underlie taurodontism in a range of syndromes.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

SCHOLARONE™  
Manuscripts

For Peer Review

# The impact of the Eda pathway on root development

Juan M. Fons Romero<sup>1</sup>, Haza Star<sup>1</sup>, Rupali Lav<sup>1</sup>,  
Sophie Watkins<sup>2</sup>, Mike Harrison<sup>2</sup>, Maria  
Hovorakova<sup>3</sup>, Denis Headon<sup>4</sup>, Abigail S. Tucker<sup>1</sup>§

<sup>1</sup>Department of Craniofacial Development and Stem Cell Biology, King's College London, Floor 27 Guy's Tower, Guy's Hospital, London Bridge, London, SE1 9RT.

<sup>2</sup>Hypodontia Clinic, Guy's & St Thomas' NHS Foundation Trust, Floor 26 Guy's Tower, Guy's Hospital, London Bridge, London SE1 9RT.

<sup>3</sup>Department of Developmental Biology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic.

<sup>4</sup>The Roslin Institute and Royal (Dick) School of Veterinary Studies University of Edinburgh, Edinburgh, United Kingdom

§ author for Correspondence

abigail.tucker@kcl.ac.uk

Keywords: Tooth root, Taurodont, furcation, Proliferation, HERS.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

Abstract:

The *Eda* pathway (*Eda*, *Edar*, *Edaradd*) plays an important role in tooth development, determining tooth number, crown shape and enamel formation. Here we show that the *Eda* pathway also plays a key role in root development. *Edar* (the receptor) is expressed in Hertwig's Epithelial Root sheath (HERS) during root development with mutant mice showing a high incidence of taurodontism: large pulp chambers lacking or showing delayed bifurcation or trifurcation of the roots. The mouse upper second molars in the *Eda* pathway mutants show the highest incidence of taurodontism, this enhanced susceptibility being matched in human patients with mutations in *EDA-A1*. These taurodont teeth form due to defects in the direction of extension of the HERS from the crown, associated with a more extensive area of proliferation of the neighbouring root mesenchyme. In those teeth where the angle at which the HERS extends from the crown is very wide and therefore more vertical, the mutant HERS fail to reach each other in the normal furcation region and a taurodont tooth is created. The phenotype is variable, however, with milder changes in angle and proliferation leading to normal or delayed furcation. This is the first analysis of the role of *Eda* in the root, showing a direct role for this pathway during postnatal mouse development, and suggests that changes in proliferation and angle of HERS may underlie taurodontism in a range of syndromes.

## Introduction:

The root of a tooth is essential for anchoring the tooth germ in place. Defects in root size and shape can lead to problems with attachment, and impact on the rest of the periodontium, therefore an understanding of root development is essential. While crown development has been fairly intensely studied, far less research has focused on root development. From mouse knockout studies a number of signalling pathways and transcription factors have been indicated as being essential for normal root development, such as *Shh*, *Wnt*, *Bmp*, *Tgfb* and *Nfic* (Li et al., 2017). These pathways are involved in complex signalling between the epithelium of the root, known as Hertwig's Epithelial Root Sheath (HERS), and the overlying pre-odontoblast neural crest derived mesenchyme. Loss of *Smad4* (acting downstream of *Tgfb* signalling) in the HERS leads to a failure in all root development (Huang et al., 2010), while loss of *Smad4* in the mesenchyme alone leads to shorter roots (Gao et al., 2009). *Smad4* appears to act upstream of *Shh*, as altering Sonic signalling can alleviate some of the defects in the *Smad4* knockout (Huang et al., 2010), with *Shh* switching on *Nfic* in the root mesenchyme and acting in a negative feedback loop (Huang et al., 2010; Liu et al., 2015).

HERS is formed as an extension of the cervical loops at the late bell stage of development. The sheath is formed of two layers, at the junction between the outer and inner dental epithelium. In the mouse this process starts at postnatal day four, with extension of this double-layered structure (Lungova et al., 2011). The HERS then extend downwards to create the roots, stimulating differentiation of the surrounding dental papilla mesenchyme to differentiate as odontoblasts and secrete the

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

dentin of the root. In single rooted teeth the HERS extend downwards in a sheet, however to generate multiple roots the HERS must change direction and extend horizontally to create furcations. The creation of these epithelial diaphragms has been proposed to be controlled by proliferation of the adjacent mesenchyme, with higher proliferation pushing the HERS to extend more vertically, while lower proliferation allows the HERS to bend inwards to divide the roots (Sohn et al., 2014). In keeping with this find, altered proliferation in the presumptive bifurcation regions have been identified in mouse mutants with root defects (Kim et al., 2015).

The *Eda* pathway has not been investigated in any detail during root development. The pathway consists of the ligand *Ectodysplasin (Eda)*, the receptor (*Edar*), a Tumour necrosis factor (TNF) family member, and intracellular adapter protein (*Edaradd*). *Eda* is cleaved to form a soluble ligand that can then bind to *Edar*, recruits *Edaradd*, and ultimately stimulates signalling through NfKappa B (Courtney et al., 2005). During early development *Eda* is expressed in the forming dental placodes, with smaller placodes forming in *Eda* mutants (known as Tabby mutants) (Pispa et al., 1999). Intriguingly these mice can also have supernumerary teeth formed from revitalisation of diastema tooth buds so both hypodontia and hyperdontia can be features in the mouse (Prochazka et al., 2010)(Charles et al., 2009b). At the cap stage the *Eda* pathway plays a crucial role in molar crown formation with pathway mutants having molars with a reduced number of flattened cusps (Gruneberg, 1966; Pispa et al., 1999; Tucker et al., 2000). This has been shown to be due to defects in the primary enamel knot at the cap stage of development (Ohazama et al., 2004; Pispa et al., 1999; Tucker et al., 2000). At the cap stage *Eda* is expressed in the dental epithelium close to the oral

epithelium, while *Edar* and *Edaradd* are expressed in the enamel knot (Headon et al., 2001; Laurikkala et al., 2001; Tucker et al., 2000).

Defects in all three components of the *Eda* pathway in patients leads to Hypohidrotic ectodermal dysplasia (HED), which is characterised by defects in many ectodermally derived organs: skin, hair, sebaceous glands, sweat glands, and teeth (Headon et al., 2001; Kere et al., 1996; Monreal et al., 1999). HED is more common in males as *Eda* is located on the X chromosome, so that hemizygous males display the full phenotype. Heterozygous females with mutations in *Eda* have much milder symptoms with increased incidence of tooth agenesis (Lexner et al., 2007). Similar to the mouse mutants, patients display hypodontia (multiple missing teeth), smaller teeth, and have reduced numbers of cusps, producing peg shaped teeth (Crawford et al., 1991). In addition patients with XL-HED have root defects, including taurodontism, suggesting that the *Eda* pathway also has an important role during root development (Lexner et al., 2007). Taurodont teeth have roots that fail to bifurcate or bifurcate very late during root formation, with the result that the pulp chamber is very large at the expense of the roots. In the *Eda* mutant mouse (Tabby), variations in root pattern have also been observed, with high variation in number of roots, and possible cusp fusions, which are not always correlated with the size of the tooth crown (Charles et al., 2009a; Gruneberg, 1966).

A direct role for the *Eda* pathway in root development was suggested by the fact that *Edar* was identified, in a screen comparing molars and incisors in the rat, as a possible root-determining gene, along with other important root genes such as *Nfic* (Xing et al., 2007). We therefore decided to follow these leads and investigate root development in *Eda*



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

and *Edar* mutant mice, and compare our findings to patients with mutations in *EDA-A1* (X-linked Hypohidrotic ectodermal dysplasia).

Methods:

Patient scans:

DPT of 20 anonymised patients with confirmed *EDA-A1* mutations were obtained from the hypodontia clinic at Guy’s Hospital London. Of the 20, data of only 15 patients (aged 6-16 years) were used; the rest being excluded as the roots of the permanent molars had not developed yet. Data was provided anonymised with only age and sex provided. The project was registered with the Research and Development (R&D) department at Guy’s and St Thomas’s Hospital Trust.

Mice:

*Eda* and *Edar* mutants were mated as previously described at the Roslin Institute (Wells et al., 2010) and in the Czech republic (Peterkova et al., 2005). Pups and adult mice were culled using schedule one methods as approved by the Home office, UK. Use of animals in this project conforms with the ARRIVE guidelines.

MicroCT:

Mice were scanned using a GE Locus SP microCT scanner and a Scanco microCT 50 scanner. Classification of teeth based on extent of taurodontism was determined using both 2D planes and 3D reconstructions of individual teeth, generated using Microview software.

Histology, In situ Hybridisation and immunofluorescence:

Wax sections were used for analysis of gene expression, protein expression, histology (see supplementary methods).

Results:

### *Edar* is expressed in HERS

Although the *Eda* pathway has been followed during tooth development in a number of prenatal stages, postnatal expression in the root has not been investigated. Our first step, therefore, was to assess where (if any) *Eda* signalling was occurring in the postnatal tooth. As *Eda* as a soluble ligand we concentrated on the expression of the receptor *Edar*, which has a very restricted expression pattern in the enamel knot of the tooth at the cap stage (Fig. 1A,C). *Edar* expression, both mRNA and protein, was found to be restricted to the bilayered HERS during root development, overlapping with expression of the epithelial marker E-Cadherin (Fig. 1B,D,E). The restriction of expression to the epithelium agrees with expression of *Edar* in other ectodermal organs and suggests a direct role for this pathway in root formation. Expression levels were similar in all molar teeth.

### Taurodontism and delayed bifurcation of roots in *Eda* pathway mutants

Having shown that *Edar* is expressed in the roots we investigated the root defect in both *Eda* (Tabby) and *Edar* (Downless) mutants using microCT to produce 3D images of the molar teeth. In the mouse the first and second lower molars (M1 and M2) have two roots while the upper M1 and M2 have three roots, similar to the situation observed in humans. As

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

has been previously reported *Eda* mutants had diverse root defects, including reduced number of roots, and in a few rare cases, increased number of roots. Both *Eda* and *Edar* mutants (2 weeks and older) displayed a high incidence of taurodontism, with single roots and large pulp chambers, or delay in bifurcation, with additional variation in the length of the final root (Fig. 2A-D and data not shown). In the literature several classifications for the degree of taurodontism in humans have been described, all with problems associated with how to accurately measure the extent of the delay in bifurcation (Jafarzadeh et al., 2008). Here we used a simple system whereby we divided taurodont teeth into those with no bifurcation (known clinically as hypertaurodont, single or pyramidal depending on the shape of the root) and delayed bifurcation (known clinically as meso- or hypo- taurodontism depending on the position of the bifurcation) (N = 17 *Edar*<sup>-/-</sup> mice analysed, total of 34 M1s and 34 M2s). From our analysis the incidence of taurodontism was found to be highest in the upper jaw, compared to the lower jaw, with the second molars (M2) being more susceptible than the first molars (M1) (Table 1). In total 24.2% of upper M2s showed delayed bifurcation, with another 53% showing no bifurcation. This was in comparison to the lower M2s that showed only a 6% incidence of delayed bifurcation and a 3% incidence of single rooted teeth. Very few of the *Edar*<sup>-/-</sup> lower M1s showed a taurodont phenotype, however, the *Eda* mutants showed a higher incidence of taurodontism in the lower jaw (data not shown), agreeing with previous studies that have demonstrated subtle differences between the these two mutants (Charles et al., 2009b). At earlier stages (postnatal day 9), when the roots are still growing, microCT of *Eda* mutants revealed early defects in the formation of the roots, with some mutant teeth having an oval apical end when compared to the figure of 8 pattern in wildtype lower molars (Fig. 2E-H).

### Conserved susceptibility of upper molars in human patients

Having established that the incidence of taurodontism in *Eda* pathway mutants was very dependent on tooth type and position we decided to investigate whether similar susceptibilities were also observed in human patients with mutations in *EDA-A1*. As has been previously described patients with XL-HED (and therefore *EDA-A1* mutations) have been shown to have a high incidence of taurodontism (Crawford et al., 1991; Lexner et al., 2007). No difference in incidence of taurodontism was referred to between upper and lower teeth. Digitalized DPT (Dental panoramic tomography) scans of 15 *EDA-A1* mutant patients were collected from Guy's Hospital (Fig. 2I,J, K). Use of such scans has previously been shown to be a reliable method of detecting taurodontism in patients (Tulensalo et al., 1989). The molars of both upper and lower jaws were examined and the prevalence of taurodontism was calculated. As with the mice we divided the teeth into those that showed hypertaurodontism, single or pyramidal roots, and those that showed delayed bifurcation (both meso- and hypo- taurodont). Overall the *Eda* patients had a high incidence of taurodontisms (Table 1). Similar to the mouse, the upper molars had a higher incidence of hypertaurodont, single and pyramidal roots (54%) and delayed bifurcation (43%) compared to the lower molars, 20.5% and 4.5% respectively. Second molars also had a higher incidence of root defects than first molars in the lower jaw, although in the upper jaw the incidence of defects was similar in M1 and M2. In general, therefore, the pattern of incidence was conserved across our mouse mutants and human patients.

### HERS morphology is disrupted in *Eda* pathway mutant mice

Having established that root defects are a common feature of *Eda* pathway mutants, and patients, we aimed to investigate the underlying mechanism behind the defect. For this *Eda* mice were chosen due to their slightly higher incidence of taurodontism when compared to *Edar* mutants, with analysis focused on the lower first molar, to allow a direct comparison of root development in mutants with both taurodont and bifurcated phenotypes. The simpler root pattern of the lower molars also allowed for a clear selection of bifurcating and non-bifurcating regions in frontal sections, not possible in the trifurcating upper molars. As the phenotype was already evident by microCT at P9 (Fig. 2E-H) we turned to P7, when little root dentin has been laid down. In sections it was clear that all the mutant teeth were narrower than the WT's (Fig. 3A,B compared to C-F), agreeing with the smaller overall size of *Eda* teeth (Charles et al., 2009a). In WT's, the buccal and lingual HERS in the centre of the tooth almost contacted each other at this stage (Fig. 3A), while they remained far apart and were shorter on either side of the furcation region (Fig. 3B) (N = 3/3). In comparison the *Eda* mutants had a highly variable phenotype, with some mutants displaying shorter HERS and a large distance between the two sides of HERS throughout the tooth (Fig. 3C,D, I) (N = 5/8). In contrast, in some mutant molars the HERS almost made contact in the middle, in a similar manner to WT's (N = 3/8) (Fig. 3E,F,I). The morphology of the HERS was investigated using Keratin 14, which showed a morphologically normal looking bilayered structure in all the mutants (Fig. 3G,H).

Altered direction of HERS and proliferation in *Eda* mutants

The mutant teeth appeared more elongated with alterations in the direction that the HERS extended from the crown (Fig. 3). We therefore

analysed the angle of extension in the bifurcation region (Fig. 4A) and found the mutants had significantly wider angles with the HERS extending more vertically (Fig. 4A). Fitting with the variable incidence of taurodontism, those mutant teeth where the HERS had managed to almost reach the midline at the bifurcation region had smaller angles that were not significantly different from the WT (Fig. 4A). In contrast, the *Eda* mutants with distantly spaced HERS had much larger and therefore more vertical HERS (Fig. 4A). In *Nfic* mutants, root furcation defects have been linked to changes in proliferation of the mesenchyme adjacent to the HERS (Kim et al., 2015), and differential proliferation has been suggested to influence the direction of HERS in wildtype roots (Sohn et al., 2014). We therefore investigated proliferation in our *Eda* mutants compared to WT (N = 8 *Eda* mutant teeth, 3 WT teeth), counting in three regions associated with the HERS (Fig. 4B). In all three mesenchymal zones counted, a larger percentage of proliferating cells were observed in those mutants which failed to form a bifurcating region (Fig. 4B). In contrast, the mutants that had managed to make a bifurcation had close to WT levels of proliferation (Fig. 4B). This suggests that the difference in proliferation may drive the HERS phenotype. The epithelial HERS themselves had high levels of proliferation, matching that of the WT, although appeared shorter in the centre of the tooth.

As the *Edar* pathway is active in the HERS (Fig. 1) this change in mesenchymal proliferation suggests that the HERS signal back to the mesenchyme to control proliferation levels, and thereby the direction of root development. We therefore analysed expression of *Shh*, a key signalling factor expressed in the HERS which is known to signal to the mesenchyme (Khan et al., 2007). *Shh* has also been shown to act downstream of the *Eda* pathway in the skin and salivary glands (Pummila et al., 2007; Wells et al., 2010; Wells et al., 2011). *Shh* was expressed in

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

the HERS and in the pre-ameloblasts in the WT and a very similar pattern was observed in the *Eda* mutant teeth at P7, although levels in the pre-ameloblasts were potentially reduced (Fig. 4C). There was no clear difference between those teeth with a taurodont or normal bifurcation however, suggesting that this reduction does not drive the phenotype.

Discussion:

Here we show for the first time that a component of the *Eda* pathway, *Edar*, is expressed during root development with a very specific expression in the developing HERS. Loss of *Eda* or *Edar* leads to a taurodont phenotype, with the upper second molars being the most sensitive to loss of this pathway. Fitting with the variable phenotype in adults, during root development *Eda* mutant molars could be divided into those with normal proliferation, HERS angle and formation of a bifurcation, and those where proliferation was increased in the mesenchyme and the HERS extended in a more vertical direction. Changes in proliferation therefore appear to drive the phenotype, agreeing with the hypothesis that uniform high proliferation throughout the root mesenchyme leads to a lack of furcation (Kim et al., 2015). It would be interesting therefore to assess whether uniform proliferation, underlies taurodontism in other syndromes and mutant mice.

As *Edar* was expressed in the HERS, this suggests another signalling factor produced by the HERS was altered in the absence of active *Eda* signalling. One candidate linked to the *Eda* pathway in other organs was *Shh*, however no clear change in *Shh* in the HERS was observed, although there might be subtle differences in levels of expression. Another candidate could be a member of the Wnt signalling pathway, as *Axin2*, a target of Wnt signalling, is highly expressed in the



mesenchyme adjacent to the HERS (Lohi et al., 2010), and loss and activation of the Wnt pathway leads to root defects (Bae et al., 2013; Kim et al., 2013). A taurodont phenotype is observed in patients and mice with mutations in *Wnt10a*, and loss of *Wnt10a* leads to defects in cusp pattern and hypodontia, mirroring many aspects of the *Eda* pathway mutant phenotype (Yang et al., 2015). In keeping with this, *Wnt10a* has been predicted to be a direct target of NfKappa B signalling (Krappmann et al., 2004).

Our comparison between the ratio of taurodontism in human and mutant mice revealed that there is a close similarity in the distribution of taurodontism in both samples. In both the prevalence of taurodontism was higher in the upper teeth compared to the lower teeth. This result is consistent with the findings that many tooth anomalies, such as taurodontism, hypodontia, and dens invagination, are more common in the upper jaws compare to the lower jaws (MacDonald-Jankowski and Li, 1993; Shokri et al., 2014). These differences in susceptibility may be influenced by the larger number of roots that form in the upper molars in both mice and humans compared to lower molars, involving a more complex pattern of folding of the epithelium. These more complex folding patterns may be more vulnerable to changes in signalling molecules and subtle changes in proliferation. Taurodontism in general, rather than just in HED patients, is also more prevalent in second molars compared to first molars (MacDonald-Jankowski and Li, 1993). As the number of roots is the same in the first and second molars the pattern of furcation cannot explain this difference in prevalence however the time difference between the development of first and second molars may impact on the incidence of the root defect, with later developing teeth being more susceptible.



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

In conclusion we have shown that the *Eda* pathway has a direct role in root development, influencing proliferation and the angle of HERS and therefore the ability to form furcations. This pathway can therefore be added to the other signalling pathways (*Shh*, *Wnt*, *Tgfb*, *BMP*) whose role in root development is just starting to be elucidated (Li et al., 2017).

Acknowledgements:

HS was supported by the Ministry of Higher Education & Scientific research, Kurdistan region Iraq and by the Dental Institute of King’s College London. RL and JF were funded by the Wellcome Trust (102889/Z/13/Z). MH was funded by the grant agency of the Czech Republic (14-37368G). DH is supported by the BBSRC Institute Strategic Programme Grant BB/J004316/1 to the Roslin Institute. Thanks to Chris Healy for help with microCT funded by a multi-user equipment grant from the Wellcome Trust grant (202852/Z/16/Z). Thanks to Max Smith for angle measurements. The authors declare that they have no conflict of interest.

Figure legends:

Figure 1: *Edar* is expressed in HERS during root development.

(A,B) In situ hybridisation for *Edar*, positive stain in blue. (A) E (Embryonic day) 15.5 late cap stage tooth. Expression of *Edar* is observed in the dental epithelium, in the enamel knot (arrow) and spreading out across the inner dental epithelium, particularly on the lingual side of the tooth. (B) At P (postnatal day) 9 *Edar* is expressed in the HERS (arrowed) at the apical end of the tooth. (C,D) Immunofluorescence for *Edar*. Signal in red, nuclei labelled by DAPI (blue). (C) High power view of the primary enamel knot (arrow) at E15.0. (D) High power of HERS (arrows) at P9, at the base of the developing tooth. (E) Serial section to (D) showing immunofluorescence for E-Cadherin, a marker of epithelium overlapping with *Edar* in the HERS. Scale in A, B: 200µm. C: 100µm. D: 50µm (same scale in E).

Figure 2: *Eda* mouse mutants and patients with *EDA-A1* mutations have a taurodont phenotype

(A, C, E-H) MicroCT 3D reconstructions of *Eda* mutant molar mice teeth. (B,D) Trichrome stained histology sections through adult erupted second molar. (A-D) 6 month old adult teeth. (E-H) P9 teeth. (A,B, E, F) Wild type teeth. (C,D,G,H) *Eda* mutant molars. (A,B) WT upper molar with three roots (arrowed), 2 root visible in section (B). (C,D) Taurodont upper molar in *Eda* mutant with a single root and large pulp cavity. (E,F) WT lower molar forming two roots with a figure of 8 shape on the apical view (F). Arrows point to forming furcations. (G,H) *Eda* lower molar

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

with an oval shape on the apical view (H) showing no signs of forming a furcation.

(I) Digitalized DPT (Dental panoramic tomography) x-ray of a 10 year old with X-linked Hypohidrotic ectodermal dysplasia caused by a mutation in *EDA-A1* with a variable expression of the taurodont phenotype in addition to loss of multiple teeth. (J) High power of molar tooth highlighted by arrow in I displaying a taurodont phenotype. (K) Neighbouring tooth with bifurcated root.

Figure 3

HERS extension defects in *Eda* mutant mice at P7

(A-F) Trichrome stained frontal sections in the centre of the tooth (Bifurcation region) (A,C,E) and outside of the normal furcation region (B,D,F). (A,B) WT. (C-F) *Eda* mutants. \* = point where HERS have almost met in the mutant. (G,H) Keratin 14 expression in the bifurcation region in WT (G) and Mutant (H) HERS. Arrows indicate gap between HERS. (I) Graph showing *Eda* mutants can be divided into two groups dependent on the distance between the HERS at the centre of the tooth. Scale bar in A = 200 microns. Same scale in B-F. Scale bar in H = 100 microns. Same scale in G.

\* = P<0.05, \*\* = P< 0.01

Figure 4

Proliferation and angle defects in *Eda* mutant mice

(A) Angle defects in *Eda* mutant mice at P7. Histology figures showing how angle of extension was measured with an example WT and *Eda* mutant. Graph showing statistically significant changes in angle only for those mutants with defects in formation of a bifurcation when compared to WT.

Scale bar in A = 100 microns.

(B) Proliferation measured by PCNA in *Eda* mutant mice at P7. Three boxes (1,2,3) were used to measure the numbers of proliferating cells to be compared with all cells in the area. Sections showing proliferation (brown) in an example WT and *Eda* mutant. Graphs showing the difference in percentage proliferating cells in the three regions shown in the above images. Only mutants with a defect in bifurcation showed a significant difference in proliferation compared to WT.

Scale bar in B = 200 microns.

(C) Shh immunofluorescence. Confocal images with "fire luts" where blue is lowest intensity and white the highest. The expression of Shh in the HERS themselves appears similar between WT and *Eda* mutant molars.

Scale bar in C = 100 microns.

\* =  $P < 0.05$ , \*\* =  $P < 0.01$ .

Table 1: Incidence of root defects in patients with mutations in EDA-A1 and in *Edar* mutant mice.

	Upper molars			Lower molars		
	M1	M2	Total	M1	M2	Total
MICE						
Delay bifurcation %	4.55	24.2	14.4	1.5	6.1	3.8
Hypertaurodont & single root	9.1	53	31.1	0	3	1.5
PATIENTS						
Delay bifurcation %	44.8	37.5	43	0	18.2	4.5
Hypertaurodont, single root, pyramidal	51.7	62.5	54.1	16.7	36.7	20.5

## References (36)

- Bae CH, Lee JY, Kim TH, Baek JA, Lee JC, Yang X, Taketo MM, Jiang R, Cho ES. 2013. Excessive Wnt/beta-catenin signaling disturbs tooth-root formation. *Journal of periodontal research* 48(4):405-410.
- Charles C, Pantalacci S, Peterkova R, Tafforeau P, Laudet V, Viriot L. 2009a. Effect of *eda* loss of function on upper jugal tooth morphology. *Anat Rec* 292(2):299-308.
- Charles C, Pantalacci S, Tafforeau P, Headon D, Laudet V, Viriot L. 2009b. Distinct impacts of *Eda* and *Edar* loss of function on the mouse dentition. *PloS one* 4(4):e4985.
- Courtney JM, Blackburn J, Sharpe PT. 2005. The Ectodysplasin and NFkappaB signalling pathways in odontogenesis. *Archives of oral biology* 50(2):159-163.
- Crawford PJ, Aldred MJ, Clarke A. 1991. Clinical and radiographic dental findings in X linked hypohidrotic ectodermal dysplasia. *Journal of medical genetics* 28(3):181-185.
- Gao Y, Yang G, Weng T, Du J, Wang X, Zhou J, Wang S, Yang X. 2009. Disruption of *Smad4* in odontoblasts causes multiple keratocystic odontogenic tumors and tooth malformation in mice. *Molecular and cellular biology* 29(21):5941-5951.
- Gruneberg H. 1966. The molars of the tabby mouse and a test of the single-active X chromosome hypothesis. *Journal of Embryology Experimental Morphology* 15(2):223-244.
- Headon DJ, Emmal SA, Ferguson BM, Tucker AS, Justice MJ, Sharpe PT, Zonana J, Overbeek PA. 2001. Gene defect in ectodermal dysplasia implicates a death domain adapter in development. *Nature* 414(6866):913-916.
- Huang X, Xu X, Bringas P, Jr., Hung YP, Chai Y. 2010. *Smad4*-*Shh*-*Nfic* signaling cascade-mediated epithelial-mesenchymal interaction is crucial in regulating tooth root development. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 25(5):1167-1178.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

Jafarzadeh H, Azarpazhooh A, Mayhall JT. 2008. Taurodontism: a review of the condition and endodontic treatment challenges. *International endodontic journal* 41(5):375-388.

Kere J, Srivastava AK, Montonen O, Zonana J, Thomas N, Ferguson B, Munoz F, Moran D, Clarke A, Baybayan P et al. 1996. X-linked anhidrotic (hypohidrotic) ectodermal dysplasia is caused by mutation in a novel transmembrane protein. *Nature genetics* 13(4):409-416.

Khan M, Seppala M, Zoupa M, Cobourne MT. 2007. Hedgehog pathway gene expression during early development of the molar tooth root in the mouse. *Gene expression patterns : GEP* 7(3):239-243.

Kim TH, Bae CH, Lee JC, Ko SO, Yang X, Jiang R, Cho ES. 2013. beta-catenin is required in odontoblasts for tooth root formation. *Journal of dental research* 92(3):215-221.

Kim TH, Bae CH, Yang S, Park JC, Cho ES. 2015. Nfic regulates tooth root patterning and growth. *Anatomy & cell biology* 48(3):188-194.

Krappmann D, Wegener E, Sunami Y, Esen M, Thiel A, Mordmuller B, Scheidereit C. 2004. The IkappaB kinase complex and NF-kappaB act as master regulators of lipopolysaccharide-induced gene expression and control subordinate activation of AP-1. *Molecular and cellular biology* 24(14):6488-6500.

Laurikkala J, Mikkola M, Mustonen T, Aberg T, Koppinen P, Pispä J, Nieminen P, Galceran J, Grosschedl R, Thesleff I. 2001. TNF signaling via the ligand-receptor pair ectodysplasin and edar controls the function of epithelial signaling centers and is regulated by Wnt and activin during tooth organogenesis. *Developmental biology* 229(2):443-455.

Lexner MO, Bardow A, Hertz JM, Nielsen LA, Kreiborg S. 2007. Anomalies of tooth formation in hypohidrotic ectodermal dysplasia. *International journal of paediatric dentistry* 17(1):10-18.

Li J, Parada C, Chai Y. 2017. Cellular and molecular mechanisms of tooth root development. *Development* 144(3):374-384.

Liu Y, Feng J, Li J, Zhao H, Ho TV, Chai Y. 2015. An Nfic-hedgehog signaling cascade regulates tooth root development. *Development* 142(19):3374-3382.

Lohi M, Tucker AS, Sharpe PT. 2010. Expression of Axin2 indicates a role for canonical Wnt signaling in development of the crown and root during pre- and postnatal tooth development. *Developmental dynamics : an official publication of the American Association of Anatomists* 239(1):160-167.

Lungova V, Radlanski RJ, Tucker AS, Renz H, Misek I, Matalova E. 2011. Tooth-bone morphogenesis during postnatal stages of mouse first molar development. *Journal of anatomy* 218(6):699-716.

MacDonald-Jankowski DS, Li TT. 1993. Taurodontism in a young adult Chinese population. *Dento maxillo facial radiology* 22(3):140-144.

Monreal AW, Ferguson BM, Headon DJ, Street SL, Overbeek PA, Zonana J. 1999. Mutations in the human homologue of mouse dl cause autosomal recessive and dominant hypohidrotic ectodermal dysplasia. *Nature genetics* 22(4):366-369.

Ohazama A, Courtney JM, Tucker AS, Naito A, Tanaka S, Inoue J, Sharpe PT. 2004. Traf6 is essential for murine tooth cusp morphogenesis. *Developmental dynamics : an official publication of the American Association of Anatomists* 229(1):131-135.

Peterkova R, Lesot H, Viriot L, Peterka M. 2005. The supernumerary check tooth in tabby/EDA mice- a reminiscence of the premolar in mouse ancestors. *Arch. Oral Biol.* 50 (2): 219-225.

Pispa J, Jung HS, Jernvall J, Kettunen P, Mustonen T, Tabata MJ, Kere J, Thesleff I. 1999. Cusp patterning defect in Tabby mouse teeth and its partial rescue by FGF. *Developmental biology* 216(2):521-534.

Prochazka J, Pantalacci S, Churava S, Rothova M, Lambert A, Lesot H, Klein O, Peterka M, Laudet V, Peterkova R. 2010. Patterning by heritage in mouse molar row development. *Proceedings of the National Academy of Sciences of the United States of America* 107(35):15497-15502.

Pummila M, Fliniaux I, Jaatinen R, James MJ, Laurikkala J, Schneider P, Thesleff I, Mikkola ML. 2007. Ectodysplasin has a dual role in ectodermal organogenesis: inhibition of Bmp activity and induction of Shh expression. *Development* 134(1):117-125.

Shokri A, Poorolajal J, Khajeh S, Faramarzi F, Kahnamoui HM. 2014. Prevalence of dental anomalies among 7- to 35-year-old people in



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

Hamadan, Iran in 2012-2013 as observed using panoramic radiographs. *Imaging science in dentistry* 44(1):7-13.

Sohn WJ, Choi MA, Yamamoto H, Lee S, Lee Y, Jung JK, Jin MU, An CH, Jung HS, Suh JY et al. 2014. Contribution of mesenchymal proliferation in tooth root morphogenesis. *Journal of dental research* 93(1):78-83.

Tucker AS, Headon DJ, Schneider P, Ferguson BM, Overbeek P, Tschopp J, Sharpe PT. 2000. Edar/Eda interactions regulate enamel knot formation in tooth morphogenesis. *Development* 127(21):4691-4700.

Tulensalo T, Ranta R, Kataja M. 1989. Reliability in estimating taurodontism of permanent molars from orthopantomograms. *Community dentistry and oral epidemiology* 17(5):258-262.

Wells KL, Mou C, Headon DJ, Tucker AS. 2010. Recombinant EDA or Sonic Hedgehog rescue the branching defect in Ectodysplasin A pathway mutant salivary glands in vitro. *Developmental dynamics : an official publication of the American Association of Anatomists* 239(10):2674-2684.

Wells KL, Mou C, Headon DJ, Tucker AS. 2011. Defects and rescue of the minor salivary glands in Eda pathway mutants. *Developmental biology* 349(2):137-146.

Xing X, Deng Z, Yang F, Watanabe S, Wen L, Jin Y. 2007. Determination of genes involved in the early process of molar root development initiation in rat by modified subtractive hybridization. *Biochemical and biophysical research communications* 363(4):994-1000.

Yang J, Wang SK, Choi M, Reid BM, Hu Y, Lee YL, Herzog CR, Kim-Berman H, Lee M, Benke PJ et al. 2015. Taurodontism, variations in tooth number, and misshapened crowns in Wnt10a null mice and human kindreds. *Molecular genetics & genomic medicine* 3(1):40-58.

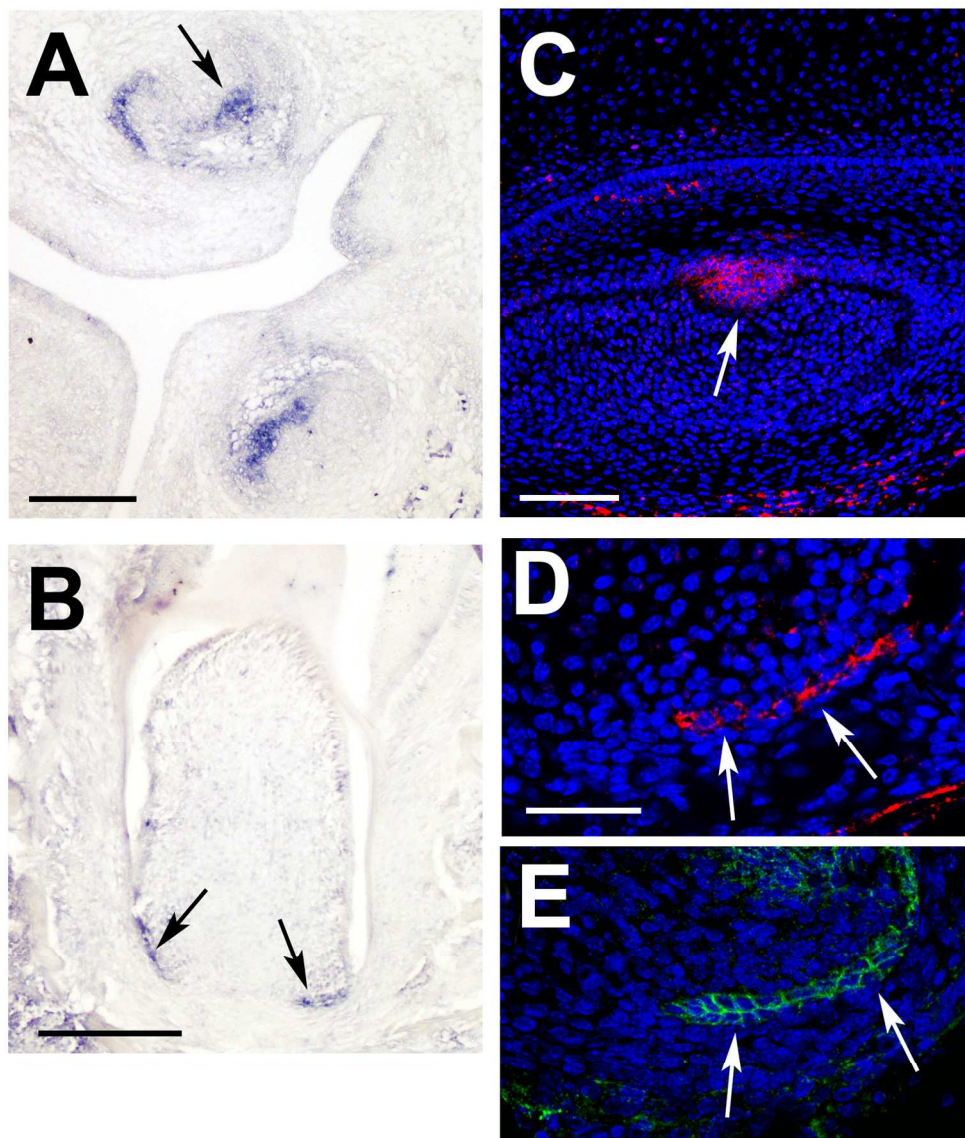


Figure 1

146x171mm (300 x 300 DPI)

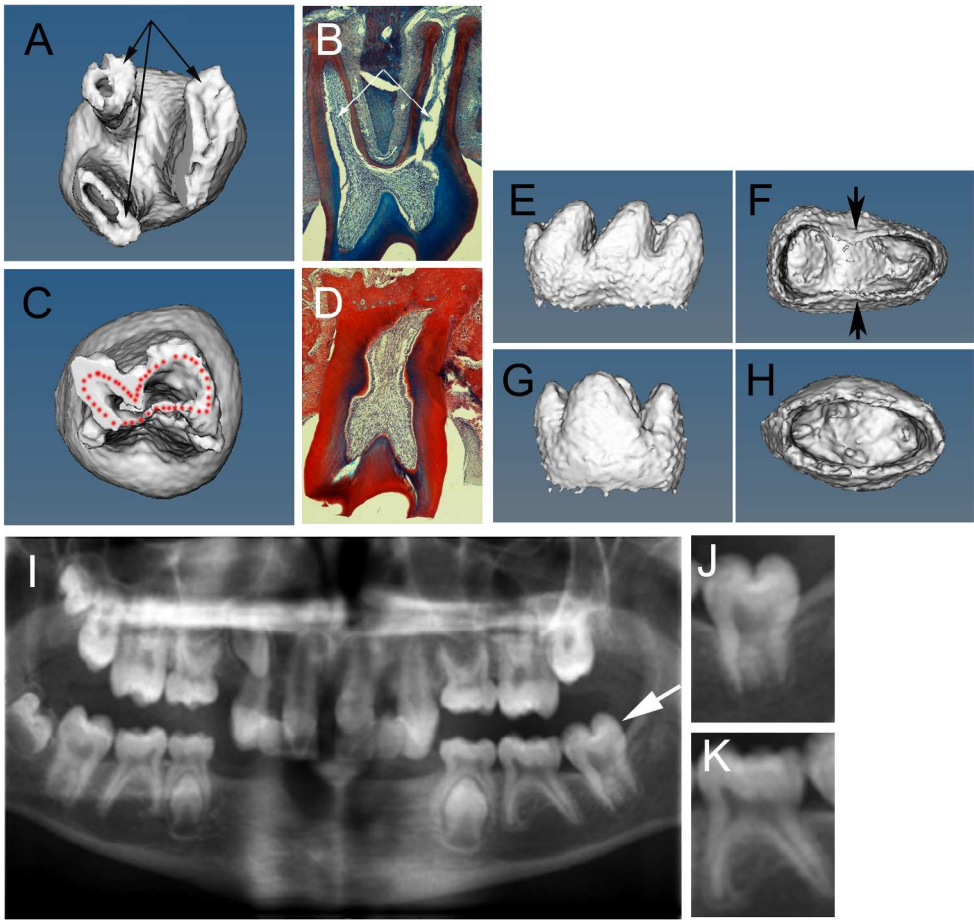


Figure 2

mc.manuscriptcentral.com/jdr



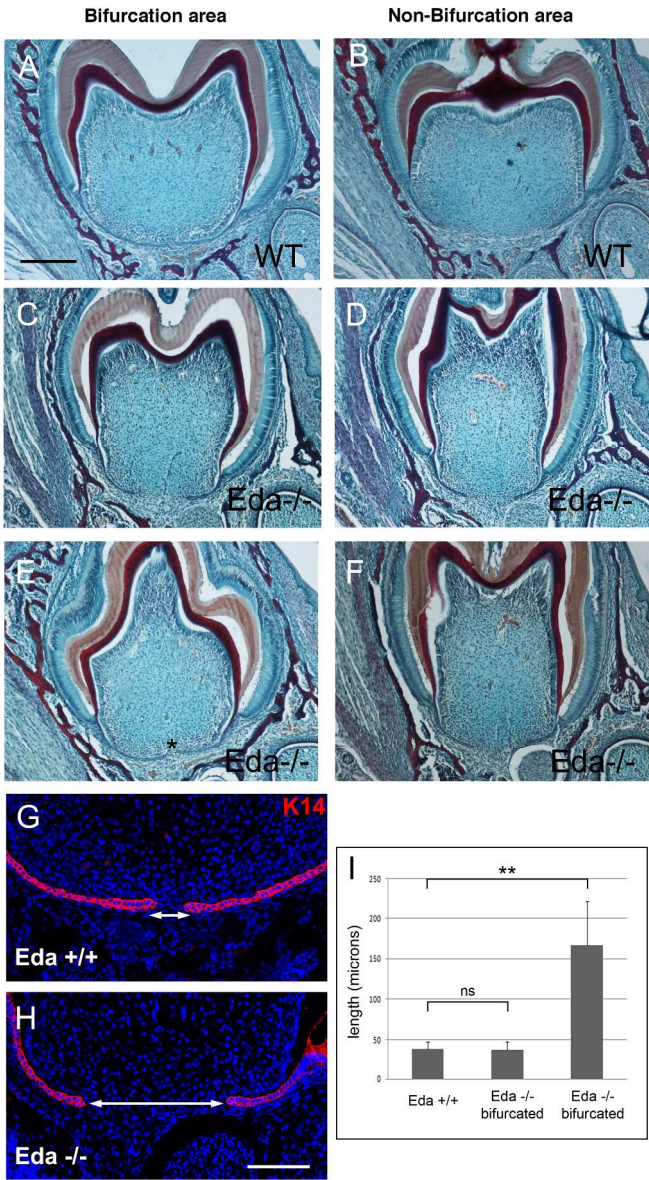


Figure 3

180x323mm (300 x 300 DPI)

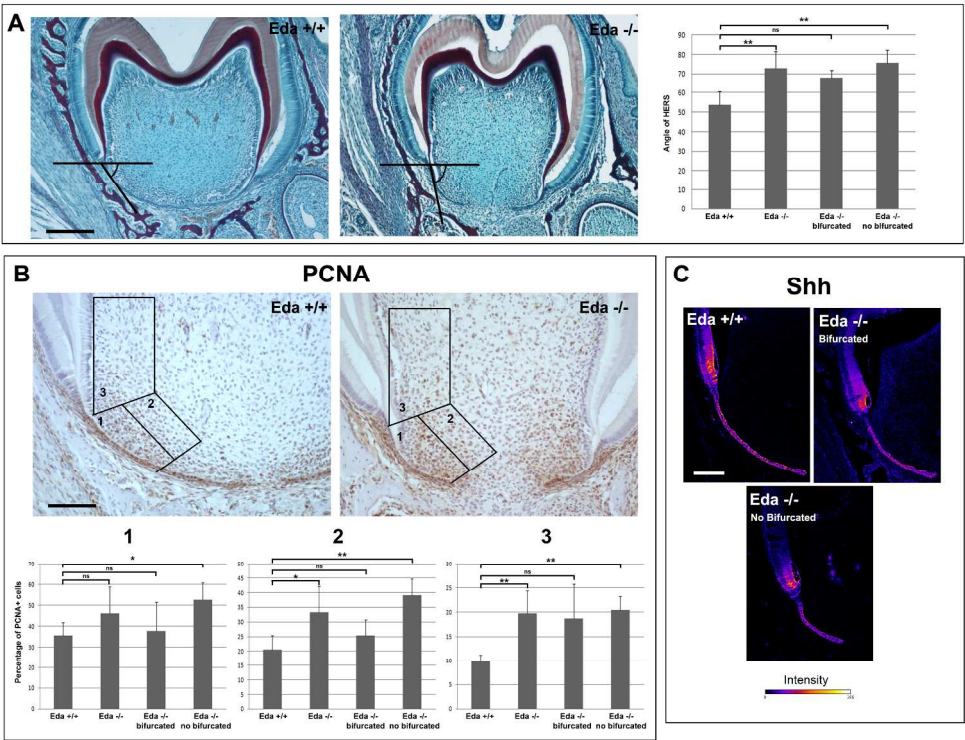


Figure 4

1062x812mm (72 x 72 DPI)

## Supplementary methods:

### Samples:

Postnatal heads were divided into upper and lower jaws, fixed in 4% paraformaldehyde and decalcified using EDTA, time depending on age of the specimen. Embryonic heads were fixed whole and did not require a decalcification step. After decalcification/fixation samples were dehydrated through an ethanol series before embedding in paraffin via histoclear. For histology a trichrome stain was used involving alcian blue, sirrus red and haematoxylin.

### In situ hybridization:

For in situ hybridization, sections were rehydrated, refixed in PFA 4% for 20min, permeabilized in 10ug/ml Proteinase K for 8 minutes, acetylated in triethanolamine (T58300 Sigma-Aldrich) plus acetic anhydride (100022M BDH) for 10 minutes and dehydrated again prior to the addition of anti-sense probe. The *Edar* probe was used at 1ug/ml and added in hybridization buffer (50% formamide, 20mM Tris/DEPC pH 7.5, 300mM NaCl/DEPC, 5mM EDTA/DEPC, 1x Denhardt's solution, 10% dextran sulphate, 0.5mg/ml tRNA) and incubated at 60°C O.N. Samples were washed in 50%formamide-2x SSC, 2xSSC and 0.2xSSC each one twice for 30min at 60°C. Sections were washed in TN buffer (100mM Tris pH 7.5, 150mM NaCl) and blocked in TN buffer with 10% fetal bovine serum (F90665, Sigma) plus 1% BBR (Boehringer, 1096176) for 1 hour-RT and incubated with 1:1000 anti-DIG Alkaline Phosphatase antibody (Boehringer, 1093274) overnight at 4 °C in blocking solution. Samples were washed in TN buffer for 1 hour and incubated in NTMT

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

(100 mM Tris-HCl pH 9.5, 50 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.1% Tween 20) twice for 10 minutes. The colour reaction was developed with BM purple (11 442 074 001, Roche). Slides were then dehydrated and coverslipped using depex. Images were taken on a Nikon light microscope.

Immunofluorescence:

For immunofluorescence, sections were rehydrated and treated with Tris-EDTA pH9 for 30 minutes at 90°C for antigen retrieval. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 30min. Sections were blocked in TN buffer plus 0.5% BBR, 10% Serum, 1% BSA and 0.05% Tween 20 for 1h at RT. Anti-Edar (Santa Cruz, sc-15289) or Anti-Shh (Santa Cruz,sc-9024) were added at 1:200 in blocking solution ON at 4°C. After washing in PBS 0.05% Tween 20, a biotinilated secondary antibody (E0432, Dako) was added at 1:300 for 2hr at RT. followed by SA-HRP at 1/150 and 1/150 TSA Fluorophore (Cy3). Slides were mounted with Fluoroshield™ DAPI (ab104139, Abcam). For Ecad (Abcam, ab76319) and K14 (Abcam, ab7800) detection, a secondary Alexa-488 was used. Slides were imaged on a confocal microscope. Shh images used “fire luts” to indicate intensity where blue is the lowest and white the highest.

Proliferation:

Detection of proliferative cell nuclear antigen (PCNA) was achieved using anti-PCNA-Biotin (Abcam, ab113270) at 1/400 followed by SA-HRP at 1/250. The signal was developed using Vector DAB staining kit (SK-4100). Slides were weakly counterstained with

Haematoxylin before dehydration and coverslipping. Images were taken on a Nikon light microscope.

#### Measurements and statistics:

For consistency all tooth measurements were made of the HERS on the buccal side of the tooth. Tooth angle measurements were made as illustrated in Figure 4A. Measurements were made by a researcher blind to the genotype of the tooth with no knowledge of HERS development to ensure unbiased recording. Proliferating cells were counted in different regions adjacent to the HERS as depicted in Fig4B and compared to total number of cells to give a percentage proliferation. Unpaired T-tests were performed with one tail and unequal variance for comparing the distance between the HERS (Fig3I), or two tails and unequal variance for the rest of the statistical analysis. Unequal variance was used due to the large variation between mutant molars compared to very small variation between controls.

#### microCT:

Specimens were immobilized using cotton gauze and scanned to produce 14µm voxel size volumes, using an X-ray tube voltage of 80kVp and a tube current of 80µA. An aluminum filter (0.05mm) was used to adjust the energy distribution of the X-ray source. To ensure scan consistency, a calibration phantom of known geometry (a dense cylinder) was positioned within the field of acquisition for each scan. Test reconstructions on this object were carried out to determine the optimum conditions for reconstruction, ensuring consistency in image



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

quality, and minimising blurring.

For Peer Review

Table 1: Incidence of root defects in patients with mutations in EDA-A1 and in *Edar* mutant mice.

	Upper molars			Lower molars		
	M1	M2	Total	M1	M2	Total
MICE						
Delay bifurcation %	4.55	24.2	14.4	1.5	6.1	3.8
Hypertaurodont & single root	9.1	53	31.1	0	3	1.5
PATIENTS						
Delay bifurcation %	44.8	37.5	43	0	18.2	4.5
Hypertaurodont, single root, pyramidal	51.7	62.5	54.1	16.7	36.7	20.5

# The ARRIVE Guidelines Checklist

## Animal Research: Reporting In Vivo Experiments

Carol Kilkenny<sup>1</sup>, William J Browne<sup>2</sup>, Innes C Cuthill<sup>3</sup>, Michael Emerson<sup>4</sup> and Douglas G Altman<sup>5</sup>

<sup>1</sup>The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, <sup>2</sup>School of Veterinary Science, University of Bristol, Bristol, UK, <sup>3</sup>School of Biological Sciences, University of Bristol, Bristol, UK, <sup>4</sup>National Heart and Lung Institute, Imperial College London, UK, <sup>5</sup>Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	
INTRODUCTION			
Background	3	<p>a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale.</p> <p>b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.</p>	
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	
Study design	6	<p>For each experiment, give brief details of the study design including:</p> <p>a. The number of experimental and control groups.</p> <p>b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when).</p> <p>c. The experimental unit (e.g. a single animal, group or cage of animals).</p> <p>A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.</p>	
Experimental procedures	7	<p>For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example:</p> <p>a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s).</p> <p>b. When (e.g. time of day).</p> <p>c. Where (e.g. home cage, laboratory, water maze).</p> <p>d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).</p>	
Experimental animals	8	<p>a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range).</p> <p>b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.</p>	

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	
<b>RESULTS</b>			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50% <sup>2</sup> ). b. If any animals or data were not included in the analysis, explain why.	
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	
<b>DISCUSSION</b>			
Interpretation/scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results <sup>2</sup> . c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	
Generalisability/translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	

## References:

- Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
- Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.